

# Microbial dynamics in upflow anaerobic sludge blanket (UASB) bioreactor granules in response to short-term changes in substrate feed

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The upflow anaerobic sludge blanket (UASB) reactor is a microcosm for the methanogenic degradation of organic matter in anaerobic environments, and depends on the auto-formation of dense 3D biofilms of 1–3 mm in diameter, referred to as granular sludge (biogranules). Past research has shown that UASB and other methanogenic reactors are extremely stable functionally, but the underlying basis of the functional stability is not well understood. In this study, microbial dynamics in the communities residing in UASB biogranules were analysed to determine responses to short-term perturbations (change in reactor feed). The reactor was fed with simulated brewery wastewater (SBWW) for 1.5 months (phase 1), acetate/sulfate for 2 months (phase 2), acetate alone for 3 months (phase 3) and then a return to SBWW for 2 months (phase 4). Analysis of 16S rRNA, methanogen-associated *mcrA* and sulfate reducer-associated *dsrAB* gene-based-clone libraries showed a relatively simple community composed mainly of the methanogenic archaea (*Methanobacterium* and *Methanosaeta*), members of the green non-sulfur (*Chloroflexi*) group of bacteria and *Syntrophobacter*, *Spirochaeta*, *Acidobacteria* and *Cytophaga*-related bacterial sequences. The *mcrA* clone libraries were dominated throughout by *Methanobacterium*- and *Methanospirillum*-related sequences. Although the reactor performance remained relatively stable throughout the experiment, community diversity levels generally decreased for all libraries in response to a change from SBWW to acetate alone feed. There was a large transitory increase noted in 16S diversity at the 2 month sampling on acetate alone, entirely related to an increase in bacterial diversity. Upon return to SBWW conditions in phase 4, all diversity measures returned to near phase 1 levels. Our results demonstrated that microbial communities, even highly structured ones such as in UASB biogranules, are very capable of responding to rapid and major changes in their environment.

Received 26 November 2009

Revised 27 April 2010

Accepted 28 April 2010

## INTRODUCTION

The majority of microbial life in nature exists as groups of interdependent, highly cooperative and functionally

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**Abbreviations:** ATM, acetotrophic methanogens; COD, chemical oxygen demand; GNS, green-non-sulfur; HTM, hydrogenotrophic methanogens; OTU, operational taxonomic unit; SBWW, synthetic brewery wastewater; UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are AY940120.1–AY940126.1, AY899826.1–AY899846.1, AY910845.1–AY910860.1, AY903645.1–AY903697.1, AY937274.1–AY937285.1 and AY929594.1–AY929610.1.

Three supplementary figures and six supplementary tables are available with the online version of this paper.

distinct organisms living in spatially well-defined communities such as biofilms and flocs (Davey & O'Toole, 2000; Sekiguchi *et al.*, 2001a; Briones & Raskin, 2003). The close association of different types of organisms offers advantages to the individuals living in the community: protection from toxins, predators and invasive species, the concentration and sequestering of limiting nutrients (for example N and P, metals), the acquisition of new genetic traits by horizontal gene transfer, plus the ability to derive energy and carbon from recalcitrant or complex substrates, overcoming thermodynamic barriers to utilize compounds in syntrophic fashion for the good of the whole community. An example of a highly structured community of micro-organisms is found in upflow anaerobic sludge blanket (UASB) reactors, the most widely used process configuration in the world for anaerobic treatment of industrial wastes (Lettinga, 1995; Sekiguchi *et al.*, 2001a; Diaz *et al.*, 2003). The UASB reactor is a microcosm for

the methanogenic degradation of organic matter in anaerobic environments, and depends on the auto-formation of dense 3D biofilms of 1–3 mm in diameter, referred to as granular sludge (biogranules). The composition of the bacterial communities residing in biogranules depends on the waste being treated, but generally consists of two or three distinct trophic levels of organisms, such as fermentative organisms, fatty acid-oxidizing bacteria (syntrophs) and acetogenic bacteria (Schink, 1992a, 1997; Briones & Raskin, 2003). Past research has shown that UASB and other methanogenic reactors are extremely stable functionally (Fernandez *et al.*, 1999; Zumstein *et al.*, 2000; LaPara *et al.*, 2002; Pender *et al.*, 2004), but the underlying basis of the functional stability is not well understood.

The purpose of our study was to determine the effect of short-term perturbations (change in reactor feed) on the highly structured archaeal/bacterial communities residing in UASB biogranules. Our hypothesis was that the complexity and diversity of organisms should decrease in response to shifting from a more complex to a simple feeding regimen, due to the elimination of a certain trophic level. But confounding this issue is the relative physical stability and integrity of the biogranules, and the ability of fermenters and acetogens/syntrophs and other bacteria to grow by utilizing decaying biomass or by switching to alternative (as of yet unknown) energy-yielding metabolisms. The highly organized structure of the biogranules is potentially beneficial for stable community function and reactor performance, but perhaps a detriment for responding to short-term change or perturbation. To test the hypothesis, a 7 l working volume UASB reactor was seeded with biogranules from a full-scale reactor treating brewery wastewater and was fed with different substrates. At the end of each perturbation, granules were sampled and DNA was extracted for clone library analysis using (i) the 16S rRNA gene with universal primers, (ii) methanogen-associated methyl Co-M reductase gene (*mcrA*) and (iii) sulfate reducer-associated dissimilatory (bi)-sulfite reductase (*dsrAB*) genes, to focus on the functional genes of two groups most likely to be affected by the changes in substrate feed. Our results showed that microbial communities, even highly structured ones such as in UASB biogranules, are very capable of responding to rapid and major changes in their environment.

## METHODS

**Reactor operation.** The 7 l UASB reactor was set up and seeded with granules obtained from an Anheiser Busch brewery facility in Baldwinsville, NY. The system was initially fed a synthetic brewery wastewater (SBWW) developed in prior work (Wu *et al.*, 1995). The approximate contribution to the chemical oxygen demand (COD) was due to three compounds: ethanol 70 %, propionate 15 % and acetate 15 %. Nitrogen, phosphorus and sulfur were added at a ratio of COD/N/P/S of 100:1:0.2:0.2. Nitrogen, phosphorus and sulfur were supplied as urea, sodium hexametaphosphate and ferrous sulfate, respectively. Calcium and magnesium were added at

10 mg l<sup>-1</sup> for ionic balance. Dechlorinated tap water was used as the feed to which the substrates and nutrients were added via metering pumps. Trace metals essential for maintaining healthy anaerobic populations were provided via pulse addition twice per week. The reactor was operated at 35 °C with pH controlled between 6.6 and 6.8 using an online pH controller, which sends a signal to deliver a concentrated sodium bicarbonate solution. The feed (influent) COD concentration was maintained at 5600 mg l<sup>-1</sup> for all periods. This resulted in an applied organic loading rate of approximately 12 kg COD m<sup>-3</sup> per day. The system was operated to foster progressively less complex microbial communities by changing feed from SBWW, to acetate only plus the sulfur supplied as sulfate (0.35 mM), to acetate only with sulfur supplied as sulfide. To test our research hypothesis, the UASB reactor was seeded with biogranules that were fed with different substrates according to the following strategy. In phase 1, growth was on a mixture of 75 % ethanol/15 % propionate/15 % acetate/minimal sulfate (SBWW) for 1.5 months. In phase 2, the feed was switched to acetate plus minimal sulfate for 2 months, followed by phase 3 growth on acetate alone for 3 months (with a 'mid' sampling point at 2 months and 'late' sampling point at 3 months). Finally, in phase 4, the reactor was returned to the initial SBWW feed for 2 months. At the end of each perturbation, granules were sampled and DNA was extracted for clone library analysis.

**DNA isolation, PCR, cloning and sequencing.** Total DNA was isolated from approximately 200 mg granules using the UltraClean soil DNA kit according to the manufacturer's protocol (MoBio Laboratories), except initial homogenization was performed using a mini-beadbeater-8 homogenizer (Biospec Products) on homogenization setting for 3 min. The concentration of DNA was determined by using a NanoDrop ND-1000 spectrophotometer (NanoDrop) and purity was checked by agarose gel electrophoresis. Twenty-five nanograms of genomic DNA was amplified in 25 µl PCR tubes utilizing 250 µM dNTPs, 0.5–2.0 µM of each primer, 1 U Platinum *Taq* DNA Polymerase and 1 × reaction buffer (Invitrogen). A total of 35 cycles was employed, consisting of a 1 min denaturation step at 95 °C, 1 min annealing step and 1.5 min extension at 72 °C for each cycle, with an initial denaturation at 95 °C for 10 min and final extension at 72 °C for 10 min. For 16S and *dsrAB* PCR, a touchdown PCR protocol was employed (Muyzer *et al.*, 1993), decreasing the annealing temperature by 0.5 °C per cycle from 65 to 52 °C for 20 cycles, followed by 10 cycles at 52 °C. For *mcrA* PCR, 35 cycles at 52 °C constant was used for the annealing conditions. The primers used for amplification by PCR are shown in Table 1. The forward primers incorporate a G-C-rich clamp (Sheffield *et al.*, 1989), so that the PCR products can be utilized in a future DGGE study. The Arch/Bact 519FC primer was changed from the original universal primer published by Lane *et al.* (1985) to be more encompassing for the methanogenic archaeal and bacterial species expected in the granules, and less so for eukaryotic organisms. PCR products were purified using a QiaQuick gel extraction kit (Qiagen) and ligated into pSTBlue-1 vector using the Novagen perfectly blunt cloning kit (EMD Biosciences). The positive clones were sequenced by ACGT Inc. using T3 and T7 primers. The double-stranded 750–800 base DNA sequences were obtained for all *mcrA* and 16S clones. For *dsrAB* clones with insert of ~1.9 kb, the centre gap was filled with custom primers. The raw sequencing data were analysed using the Lasergene software package from DNASTAR. The sequences with less than 3 % difference at DNA level were treated as the same operational taxonomic unit (OTU). Over 600 DNA sequences were analysed in this study.

**Sequence alignment and phylogenetic analysis.** For 16S rRNA genes, the sequences were analysed using the programs from the Ribosomal Database Project II (RDP) (<http://rdp.cme.msu.edu/index.jsp>) for possible chimeras. Sequences of 16S, *mcrA* and *dsrAB* were

**Table 1.** Primers used for PCR

Name	Primer type	Sequence (5'–3')	Specificity	Reference
Arch/Bact 519FC	16S rDNA, forward clamped	CAGC(AC)GCCGC- GGTAA(TC)AC*	Archaea/bacteria specific, modified from original for this paper	Lane <i>et al.</i> (1985)
Univ1392r	16S rDNA, reverse	ACGGGCGGTGTGT(AG)C	Universal	
ME1FC	<i>mcrA</i> , forward clamped	GC(AC)ATGCA(AG)AT- (ACT)GG(AT)ATGTC*	Methyl CoM reductase, <i>mcrA</i> , methanogen specific	Hales <i>et al.</i> (1996)
ME2R	<i>mcrA</i> , reverse	TCAT(GT)GC(AG)TAGTT- (AGT)GG(AG)TAGT		
DSR1FC	<i>dsrAB</i> , forward clamped	AC(GC)CACTGGAAGCACG*	Dissimilatory sulfite reductase, <i>dsrAB</i> , sulfate reducing	Wagner <i>et al.</i> (1998)
DSR4R	<i>dsrAB</i> , reverse	GTGTAGCAGTTACCGCA	bacteria (SRB)-specific	

\*Forward G-C-rich clamp added to 5' end, sequence: CGCCCGCCGCGCCCCGCCGGCCCGCCGCCCCCGCCCGGG (Shigematsu *et al.*, 2003).

then aligned using the ARB shareware program (version 2.5b) (Ludwig *et al.*, 2004) (<http://www.arb-home.de/>). Phylogenetic trees were generated using distance (ARB and PHYLIP), parsimony (PHYLIP DNAPARS) and maximum-likelihood (fastDNAMl) methods, and bootstrapping (neighbour joining and/or parsimony) was performed. Branches with less than 50 % bootstrapping values were collapsed into higher groupings. The OTUs were then determined based on a 3 % divergence cut-off for an individual 'species' OTU. All DNA sequences have been submitted to the NCBI GenBank database.

**Statistical analysis.** Rank-based Spearman correlation coefficient was computed to indicate the microbial community similarity. In addition, the correlation was tested and a *P*-value was calculated to indicate the significance level of the correlation. The analysis was performed with SAS software (Version 9). The Shannon–Wiener index method was used to determine diversity (Shannon & Weaver, 1948). Two measures were employed to estimate the extent of coverage of diversity in our clone libraries, one based on log normal distribution (Mullins *et al.*, 1995) and another based on rarefaction analysis using a RarefactWin shareware program (<http://www.uga.edu/~strata/software/Software.html>) (Hurlbert, 1971).

## RESULTS

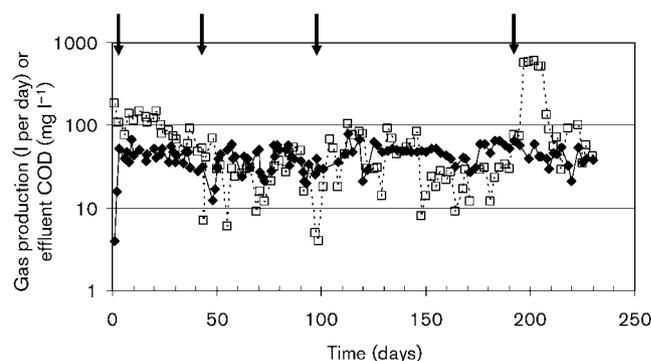
### Reactor performance

The overall performance of the 7 l UASB bioreactor is shown in Fig. 1. COD removal efficiency was generally between 97 and 100 % of substrate input, and gas production ranged between 37 and 50 litres per day on average. It is important to note that throughout the entire experiment, no macro-level physical differences were noted in biogranule appearance; they were generally round or elliptical in shape, 0.5–3 mm in diameter and black in colour.

### Dynamics of clone libraries

**16S rRNA clones.** 16S rRNA library analysis revealed a mixed community of archaeal and bacterial types (Supplementary Table S1, available with the online version of this paper). Twenty-three distinct OTUs were detected based on a 3 % sequence cut-off. Methanogens dominated the clone library at all time points (Supplementary Fig. S1),

representing nearly 75 % of all clones analysed during the experiment. Methanogenic OTUs fell into two groups, those related to *Methanobacterium subterraneum* (OTU I–III), a halotolerant methanogen isolated from deep granitic groundwater, capable of growth on hydrogen or formate (Kotelnikova *et al.*, 1998), and those related to *Methanosaeta* sp. (OTU IV–V), in which known species are capable of methanogenic growth on acetate, but not hydrogen or formate. The distribution of methanogenic clones changed during the course of the experiment. During initial growth on SBWW, OTUs related to *Methanobacterium* represented 52 % of the clones analysed, while those related to *Methanosaeta* sp. represented only 26 % of clones. After the change in feed to acetate plus sulfate, both clone types were equally represented, at 38 % of total clones. During the transition to acetate-alone feed, clone numbers related to *Methanosaeta* continued to rise, while those related to *Methanobacterium* declined, eventually

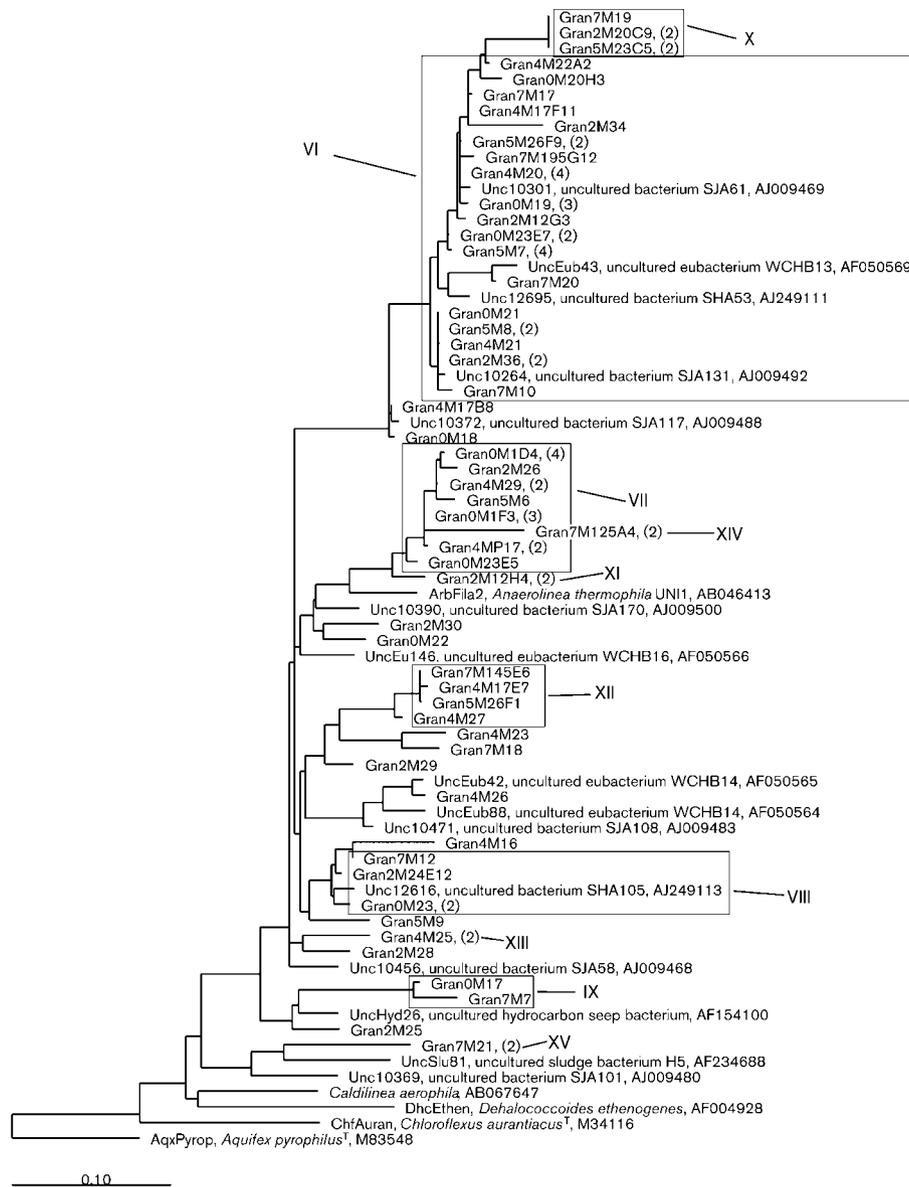


**Fig. 1.** Reactor performance. Results for gas production (l per day;  $\blacklozenge$ ) and effluent COD ( $\text{mg l}^{-1}$ ;  $\square$ ) during the course of the experiment. Arrows (from left to right) indicate major changes to substrate feed: phase 1, growth on SBWW; phase 2, change to acetate plus minimal sulfate; phase 3, change to acetate alone; and phase 4, regrowth on SBWW.

resulting in a 59 to 27% distribution, respectively. Upon return to SBWW feeding, clones related to *Methanosaeta* continued to dominate, representing 49% of clones, while those related to *Methanobacterium* represented only 22% of the clones.

The second most abundant group (11% of total clones) was related to the green-non-sulfur (GNS) group of

*Bacteria* (phylum *Chloroflexi*). Occurrences of individual OTUs fluctuated in non-discernible patterns throughout the experiment, with the group representing over 28% of total clones analysed during the 2 month sampling on acetate alone (mid). Unlike the rather monophyletic *Archaea*, the GNS group comprised many unique OTUs spanning an extensive phylogenetic distance (Fig. 2). Deltaproteobacteria related to *Syntrophobacter wolinii* were



**Fig. 2.** Phylogenetic diversity of the green-non-sulfur (GNS) group of bacteria in biogranules. The maximum-likelihood tree is based on full-length (1400+ bp) ARB-aligned sequences, with partial 16S rRNA gene biogranule clone sequences inserted into the tree using the maximum-parsimony option within ARB. OTU groups based on 3% cut-off are labelled in Roman numerals (group numbers correspond with Supplementary Table S1). Biogranule clone names refer to the sampling point (4M is a 4 month clone), with the number of closely related clones shown in parentheses. The tree was constructed with the 16S rRNA gene of *Aquifex pyrophilus* as the out-group and a filter in ARB (bac\_rr5\_june03) that excluded alignment positions with sequence ambiguity or missing data. The confidence of branch points was determined by three separate analyses (maximum-likelihood, evolutionary distance and maximum-parsimony). The scale bar indicates 10% estimated sequence divergence.

detected at most time points, but were most prevalent during the 2 month sampling on acetate alone, and during regrowth on SBWW (Supplementary Fig. S2). *S. wolinii* grows syntrophically on propionate in combination with a hydrogen-utilizing methanogen (Boone & Bryant, 1980). Clones related to *Bacteroides*, *Spirochaetes* and *Acidobacteria* were represented as well, but none was greater than 5% of the total clones analysed (Supplementary Fig. S3). Similar to the GNS group, clones from these groups fluctuated in number but were most numerous during the 2 month sampling point on acetate alone.

***mcrA* clones.** Analysis of the *mcrA* clone library revealed seven distinct clone OTUs relating to two classes of *Archaea*, the class *Methanobacteria* (OTU I–III) and the class *Methanococci* (OTU IV–VII) of the *Euryarchaeota* phylum within the domain *Archaea* (Supplementary Table S2). The distribution of individual OTUs related to *Methanobacteria* varied by treatment, with OTU I being the most numerous, and OTUs II and III fluctuating by treatment. In total, the group represented the majority of clones in the *mcrA* library, corresponding to at least two-thirds of the total clones for each treatment. The exception was the 3 month sampling on acetate alone, where clones related to *Methanobacteria* were approximately equal to those related to *Methanococci*. OTUs IV–VII of the class *Methanococci* were more divergent and each was most closely related to different organisms on the database. OTU IV, most closely related to *Methanospirillum hungatei* (Ferry *et al.*, 1974), was the most numerous clone of this type for most treatments, with the others varied by treatment. In general, *Methanospirillum*-related clones were fewer in number than those related to the *Methanobacteria*-related clones.

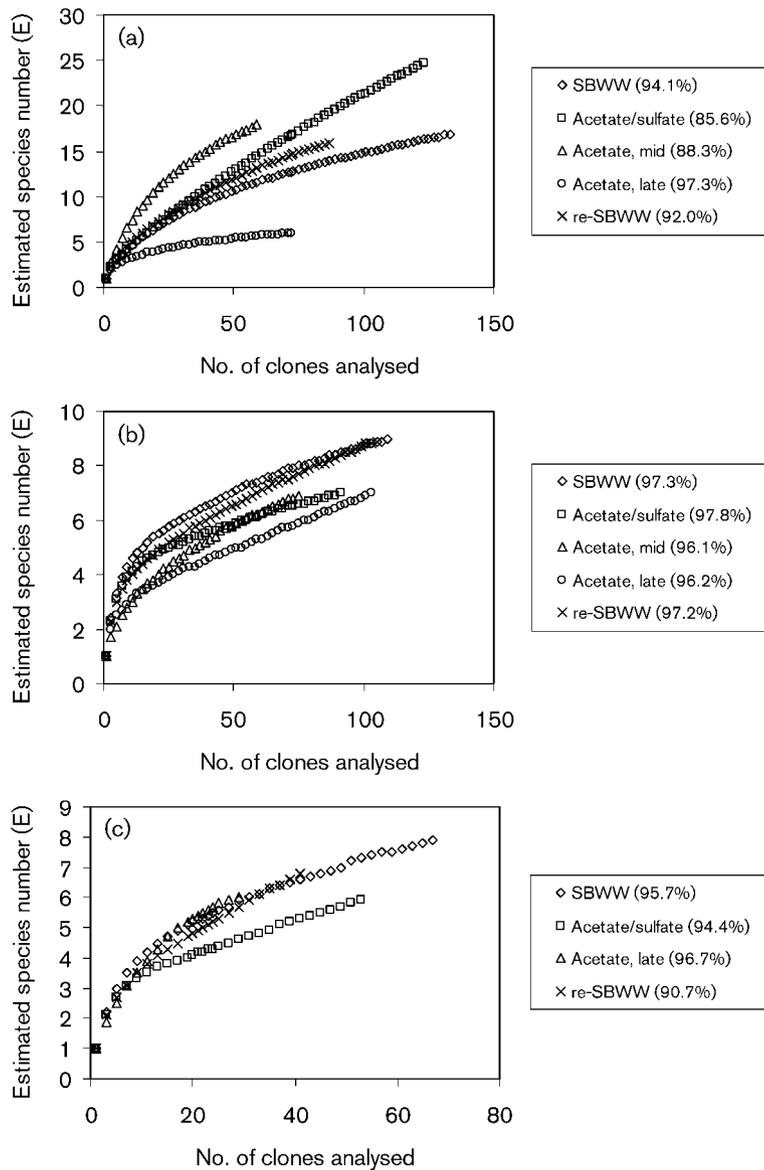
***dsrAB* clones.** Analysis of the *dsrAB* clone library revealed clones relating to 11 distinct OTUs, all related to incomplete oxidizing sulfate-reducing bacteria (Supplementary Table S3). The majority of clone types were closely related to *Desulfovibrio* species (OTU I–VI), representing 50% of all clones sampled. These clones were most numerous during the first two phases of the experiment, growth on SBWW and acetate plus sulfate, and less numerous during growth on acetate alone or during regrowth on SBWW. The second most representative clone type was closely related to *Desulfomonile tiedjei* (OTU VII), but didn't appear in our libraries until growth on acetate alone, in which it represented two-thirds of clones analysed during that treatment. The numbers for this OTU remained high during the regrowth on SBWW, representing 58% of clones sampled. Finally, there were several OTUs related to *Desulfotomaculum* species (OTU VIII–X), which were most prevalent during phase 1 growth on SBWW, representing over 50% of the clones analysed. Upon transition to the simpler feeding regimens, these clones decreased in number and were not observed with growth on acetate or regrowth on SBWW.

## Comparison of community diversity and composition

The log normal function indicated greater than 85% coverage for all libraries (range 85–98%), and the plateau shape of many of the curves indicated that the majority of the diversity was captured by our cloning effort (Tipper, 1979) (Fig. 3). The changes in community diversity, as indicated by the Shannon–Wiener diversity index, in the biogranules during the experiment are shown in Fig. 4. During the initial transition from SBWW to acetate/sulfate, there was a slight increase noted for diversity in 16S clones, while *mcrA* and *dsrAB* diversity decreased slightly. During the second transition to the acetate-alone feed, major changes in community structure occurred. At the 2 month sampling point on acetate alone (mid), the diversity of the 16S rRNA gene community rose dramatically (nearly 150% increase over initial phase 1 values), almost entirely as the result of an increased number of bacterial OTU (Fig. 4, bacterial component of the Shannon–Wiener index). At this sample point, diversity levels for the *mcrA* gene continued to decrease, paralleling changes noted for methanogens by 16S rRNA gene data (Fig. 4, archaea component of the Shannon–Wiener index). Unfortunately, we were unable to amplify DNA from the biogranules by PCR using *dsrAB* primers for this sampling point, despite repeated attempts. Since there were species present in our 16S libraries for this time point that should contain *dsrAB* genes, for example those related to the deltaproteobacterium *S. wolinii*, it was unclear why we were unable to amplify DNA from this sample. For the 3 month sampling on acetate alone (late), another dramatic shift in diversity occurred, with total 16S rRNA gene diversity dropping nearly 40% below phase 1 levels on SBWW. The diversity values for all three genes were nearly identical during this sampling (at approximately 1.6–1.7). After a return to SBWW in the final phase of our experiment, diversity measures for all groups increased to almost phase 1 levels. The similarity of microbial communities between different phases was evaluated by rank-based Spearman correlation coefficient for 16S rRNA, *mcrA* and *dsrAB* libraries (Supplementary Tables S4, S5 and S6).

## DISCUSSION

In this study, we targeted the 16S rRNA, *mcrA* and *dsrAB* genes to investigate the effect of short-term changes in substrate feed on the microbial dynamics of the highly structured archaeal/bacterial communities residing in methanogenic UASB biogranules. At the macro-level, there were no apparent differences in granule appearance, size or colour or in overall reactor performance during growth on the different substrate feeds. Even though the feed transitioned from a relatively complex one of ethanol/propionate/acetate (SBWW) to a single, simple carbon source (acetate) and then back to the more complex feed, the reactor performance remained stable throughout, as demonstrated by the constant level of COD removal and gas production (Fig. 1). COD removal efficiency was generally between 97

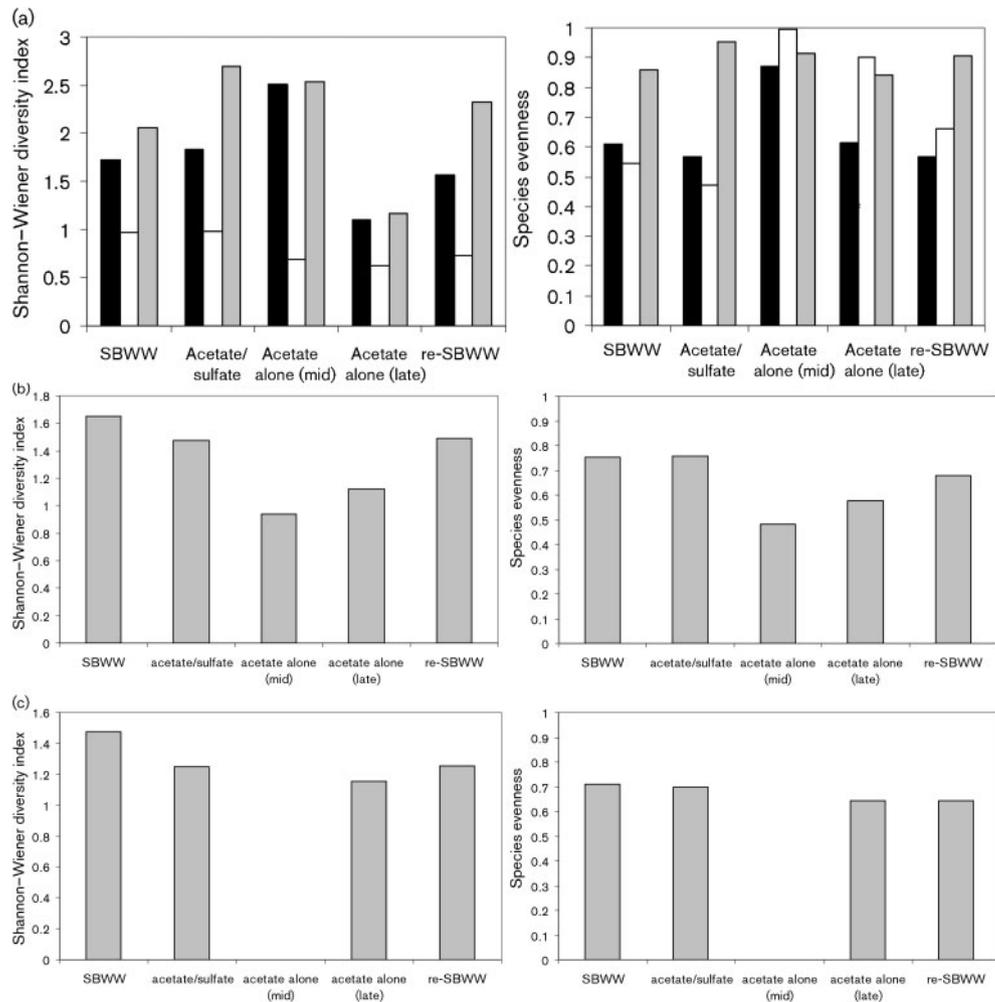


**Fig. 3.** Rarefaction analysis of clone libraries. Results of rarefaction analysis of 16S rRNA gene clones (a), *mcrA* gene clones (b) and *dsrAB* gene clones (c). The number in parentheses represents the clone coverage by the log normal method (see Methods for details).

and 99% of the input COD of  $5600 \text{ mg l}^{-1}$ . A notable decrease in removal efficiency was seen just after transition from acetate-only feed in phase 3 to phase 4 regrowth on SBWW (days 197–205 of the experiment, Fig. 1), suggesting that this transition was the most stressful one in terms of overall reactor performance, although COD removal efficiency still exceeded 90% during this time.

Although the reactor functioned in a stable manner, the microbial populations changed during the experiment. Our 16S rRNA gene, *mcrA* and *dsrAB* clone libraries all showed a general decrease in diversity during the transition from SBWW to acetate alone (Fig. 4). This was supportive of our original hypothesis that diversity would decrease due to the loss of a higher trophic level, i.e. the syntrophs and fermentative bacteria that normally convert ethanol and propionate into hydrogen and acetate utilized by the methanogens. However, there was a very large transient

increase in diversity noted in our 16S rRNA gene data for the 2 month sampling on acetate alone (mid) (value = 3.63), almost entirely related to increased numbers of OTU within the bacterial populations (Fig. 4, bacterial component of the Shannon–Wiener index). Although our rarefaction analysis indicated that we captured the majority of the diversity of organisms present with our cloning analysis (Fig. 3), the maximum saturation of potential species was never achieved for phase 2 transitions from SBWW to acetate/sulfate and acetate alone. This is consistent with prior studies in bioreactors showing large increases in rare populations of bacterial species as the result of change or perturbation (Delbes *et al.*, 2000, 2001; Zumstein *et al.*, 2000; Fernandez *et al.*, 1999, 2000; LaPara *et al.*, 2002; Pender *et al.*, 2004). Thus, the transient increase in diversity levels that was noted might be more reflective of the true complexity of bacterial populations within the structured biogranular communities.



**Fig. 4.** Changes in diversity and species evenness with treatment. Changes in Shannon–Wiener diversity index and species evenness are shown for 16S rRNA (a), *mcrA* (b) and *dsrAB* (c) genes as a result of changes in substrate feed. The relative contributions of bacterial (grey bars) and archaeal (white bars) species to the 16S rRNA gene (a) are shown (black bars, total).

The complete degradation of ethanol/propionate/acetate to  $\text{CO}_2$  and  $\text{CH}_4$  is achieved by a microbial community consisting of methanogenic archaea and fermenting and acetogenic bacteria. Ethanol can be degraded syntrophically by *Thermoanaerobium brockii*, *Peleobacter* strains and certain ethanol-oxidizing *Desulfovibrio* strains (Ben-Bassat *et al.*, 1981; Kremer *et al.*, 1988; Schink 1992a, b). From our 16S rRNA gene libraries, it was not obvious which organisms were involved in the syntrophic degradation of ethanol despite being an important component of the feed (75% of COD). Although not present in our 16S rRNA gene libraries, our *dsrAB* libraries showed that sequences related to *Desulfovibrio* (deltaproteobacteria) were present in the granules. Several *dsrAB* OTUs decreased after switching the feed to acetate alone, but seemed to be re-established after switching back to SBWW. This suggests that at least members of the species *Desulfovibrio* must have been involved in the syntrophic degradation of ethanol.

Methanogens clearly dominated the 16S rRNA gene clone libraries at all time points, but a clear distinction must be made between the two functional groups of methanogens: hydrogenotrophic (hydrogen/formate-consuming) and acetotrophic (acetate-consuming) methanogens. A shift was observed in the populations when the feed shifted from SBWW to acetate alone. Hydrogenotrophic methanogens (HTM) gave way to acetotrophic methanogens (ATM) and sequences from the latter became the dominant sequences in the 16S rRNA gene clone libraries. This can be explained by the fact that ethanol and propionate were omitted from the feed and thus no more hydrogen or formate was being produced that could be used by HTMs, resulting in ATMs taking over the clone libraries. This shift in functionality (hydrogen- vs acetate-consuming) was expected to happen and has been shown to occur in other studies (Delbes *et al.*, 2000, 2001).

Several syntrophic propionate-oxidizing bacteria have been described, such as *Smithella propionica*, *Desulfotomaculum thermobenzoicum*, *Pelotomaculum thermopropionicum* and *Syntrophobacter* species (De Bok *et al.*, 2001; Imachi *et al.*, 2002; Plugge *et al.*, 2002). One OTU related to *S. wolinii* was observed in almost all of our 16S rRNA gene libraries. The OTU disappeared in the acetate alone (late) feed but the population re-established itself as soon as the feed was switched back to the original feed with propionate. This clearly showed the involvement of *Syntrophobacter* species in the syntrophic degradation of propionate in the biogranules. Many other studies have shown that *Syntrophobacter* species are key organisms for the syntrophic oxidation of propionate in biogranules (Boone & Bryant, 1980; Wallrabenstein *et al.*, 1995; Harmsen *et al.*, 1998). However, it cannot be ruled out that other syntrophic propionate-oxidizing bacteria are of importance as well.

OTUs related to the GNS bacteria (*Chloroflexi* group) were relatively well-represented in our 16S rRNA gene libraries. Several populations restored themselves or appeared as soon as the feed was switched back to SBWW in phase 4 of our experiment (with ethanol and propionate) after the disturbance with acetate alone. This suggests that GNS bacteria might play a role in the degradation of these compounds and we therefore propose that GNS bacteria are identified as potentially syntrophic bacteria. GNS bacteria have been shown to be present in many bioreactors, but only a few have been isolated, and their exact eco-physiological role in biogranules remains largely unknown. Prior research using FISH probes specific to GNS has shown that these organisms tend to inhabit the outermost portions of biogranules (Sekiguchi *et al.*, 1999), and one isolate cultivated from a thermophilic reactor biogranule (Sekiguchi *et al.*, 2001b) was able to grow on limited substrates (sucrose, glucose or arabinose in the presence of yeast extract), producing hydrogen, acetate and CO<sub>2</sub>. Interestingly, growth was greatly increased in a co-culture with a hydrogen-consuming methanogen (*Methanothermobacter thermautotrophicus*), suggesting a syntrophic relationship with methanogens. However, to understand their exact eco-physiological role and significance in biogranules, further research is needed.

Interestingly, our *mcrA* libraries showed a different picture compared with our 16S rRNA gene libraries. In these libraries, only *mcrA* sequences from HTM were obtained, excluding sequences from ATM. Although acetate was only a small fraction of the original feed (15 % of COD), as an intermediate from ethanol and propionate it is produced in significant quantities that can almost equal 100 % of COD intrinsically. Furthermore, when the feed was switched to acetate alone (100 % of COD) a clear increase in 16S rRNA gene clones related to the acetate-consuming *Methanosaeta* species was observed, but this amplification was not reflected in the *mcrA* libraries. One possibility is the bias of the *mcrA* primers towards the *mcrA* of HTM. A recent study of *mcrA* genes using T-RFLP with defined mixtures

of methanogenic cultures showed a bias toward *Methanobacterium* sp., and against *Methanosaeta* sp., but different *mcrA* primers were used in that analysis compared with our analysis (Lueders & Friedrich, 2003). These authors speculated that the degenerate nature of the primers themselves (similar to our primers) may have contributed to the bias. The dominance of HTM in our *mcrA* libraries showed that the HTM had a great deal of resilience during and after the disturbance of their substrate feed. It is unlikely that the HTM were able to consume acetate because all of the OTUs were closely related to *Methanobacterium* species, specialists known to consume only hydrogen and formate.

Perhaps the continued presence of HTM under acetate-only feed conditions can be explained by the fact that some of the acetate may be oxidized by acetate-oxidizing syntrophs to hydrogen and carbon dioxide, which then can be utilized by the HTM. Syntrophic acetate oxidation was first described for a moderately thermophilic bacterium, strain AOR (Lee & Zinder, 1988; Zinder & Koch, 1984), and later, other syntrophic acetate-oxidizers have been described (Hattori *et al.*, 2000; Schnürer *et al.*, 1996). Although syntrophic acetate oxidation at 37 °C is presumed to be of minor importance in the biogranules because of the superior competitive advantages of acetate-consuming specialists like *Methanosaeta* species, this process may take place in biogranules. Studies have shown that different chemostat dilution rates or stress conditions caused a metabolic pathway shift between aceticlastic and non-aceticlastic methanogenesis in the degradation of acetate (Schnürer *et al.*, 1994; Shigematsu *et al.*, 2003; Wagner *et al.*, 1998). In our experiment, the switch from a complex (ethanol/propionate/acetate) to a simple feed (acetate + sulfate and acetate alone) perhaps stressed the ATM enough to make syntrophic acetate oxidation possible, or revealed its potential because microbial groups from other trophic levels started to disappear from the biogranules.

We chose DNA cloning and sequence analysis of three independent genes – rRNA gene, *mcrA* and *dsrA* – to probe the structure of the microbial communities in UASB biogranules in response to a change in reactor feed from the complex SBWW to acetate alone. Our results showed that the microbial communities changed in direct response to the feed changes, while overall reactor function remained relatively constant. Methanogen numbers dominated the 16S clone libraries, while a number of rare individual bacterial species were identified during the transition to simpler reactor feed, resulting in transitory increased diversity levels.

Further research would be required to determine the functional role of hydrogen-utilizing methanogens versus acetoclastic methanogens in the UASB biogranules in response to the change in reactor feed. An examination of mRNA changes or FISH microautoradiography analysis with radiolabelled substrates might determine whether

acetoclastic methanogens are more active during the transition to acetate feed, as indicated by our 16S data, or whether hydrogen-utilizing methanogens remain active with all feeds, as indicated by our *mcrA* data. Aside from the interesting possibility that syntrophic acetate oxidation involving many bacterial groups and hydrogen-utilizing methanogens may be involved in the conversion of acetate to methane in biogranules, the question could also have important implications for the operation of UASB reactors in general. As a near-neutral pH is one of the critical factors for reactor performance and granule integrity, different strategies (for example bioaugmentation) might be utilized to prevent build-up of volatile fatty acids and acid formation if hydrogen-utilizing methanogens were mainly responsible for methane formation.

Our clone data also indicated that the individual treatment time periods may not have been sufficient for full stabilization of the communities to equilibrium, as shown by the identification of different species at the 2 month versus 3 month time points on acetate alone. The patterns noted in our DNA-based analysis could in part also be reflective of residual DNA from dead or decaying biomass in the biogranules in response to the changes in reactor feed. Our study did not specifically address this issue, although we assumed that the processes of death and growth of various groups were occurring in response to reactor feed changes. Therefore, an analysis of mRNA would help delineate not only which archaeal and bacterial groups are most active in the biogranules but also which are growing and dying in response to reactor feed changes.

## ACKNOWLEDGEMENTS

The work was supported by a grant from Laboratory Directed Research and Development Program (LDRD) of Pacific Northwest National Laboratory (PNNL). Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the US Department of Energy through contract DE-AC06-76RLO 1830.

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Edited by: H. Daims